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Mapping of the pyrophosphate binding sites of beef heart mitochondrial F_1-ATPase by photolabelling with azidonitrophenyl [α-^{32}P]pyrophosphate

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Abstract

4-Azido-2-nitrophenyl [α-^{32}P]pyrophosphate (azido-[α-^{32}P]PP_i) mimics ADP and PP_i by some of its binding properties when assayed in the absence of photoirradiation with mitochondrial F_1-ATPase. Upon photoirradiation, both α- and β-subunits of F_1-ATPase were covalently labelled. Following chemical and enzymatic cleavages of each of the two photolabelled subunits, peptides containing the covalently bound radioactivity were separated by HPLC and identified by amino acid sequencing. Bound azido-[α-^{32}P]PP_i was found to be concentrated in two distant sequences of the α-subunit, namely Asp^{194}-Thr^{221} and Lys^{386}-Met^{437}, and in a single sequence of the β-subunit Glu^{294}-Met^{358} with most of the photoprobe bound to β-Tyr-311 and β-Tyr-345. These results are discussed in terms of a model in which the pyrophosphate binding sites of F_1 are located in regions of the α- and β-subunits exposed at the interface between the two subunits and correspond to non-catalytic and catalytic adenine nucleotide binding sites, respectively.

Keywords: Mitochondrial F_1-ATPase; ATPase, F_1-; Pyrophosphate binding site; Photoaffinity

1. Introduction

Besides its adenine nucleotide binding sites and P_i binding sites, mitochondrial F_1-ATPase contains three pyrophosphate (PP_i) binding sites which exhibit high affinity and specificity for PP_i [1]. PP_i binding to mitochondrial F_1 influences the binding of nucleotides to both the catalytic and non-catalytic sites [2]. In a preliminary study, the synthesis of a radiolabelled azido derivative of PP_i, 4-azido-2-nitrophenyl [α-^{32}P]pyrophosphate (azido-[α-^{32}P]PP_i), was described. Its affinity and specificity, investigated in the absence of photoirradiation, made this derivative a good probe for mapping studies of the PP_i binding sites in mitochondrial F_1 [3]. In the present report, we describe photolabelling experiments that led to the identification of short amino acid sequences in the α- and β-subunits that are covalently labelled by azido-[α-^{32}P]PP_i.

2. Materials and methods

2.1. Materials

The chemicals and their sources were as follows: trypsin, Worthington; *S. aureus* V_8 proteinase, Miles; endo Asp-N proteinase (sequencing grade), Boehringer; Sequelon arylamine, Millipore; EDC, Sigma; molecular weight markers for SDS-PAGE, BDH. Azido-[α-^{32}P]PP_i was synthesized and purified as described in [3]. Beef heart mitochondrial

Abbreviations: Azido-[α-^{32}P]PP_i, 4-azido-2-nitrophenyl [α-^{32}P]pyrophosphate; Azido-[α-^{32}P]P_i, 4-azido-2-nitrophenyl [α-^{32}P]phosphate; CHAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CNBr, cyanogen bromide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; F_1, catalytic sector (soluble) of the beef heart mitochondrial ATPase; PAGE, polyacrylamide gel electrophoresis; PP_i, pyrophosphate; TDAB, tetradecyltrimethyl ammonium bromide; TFA, trifluoroacetic acid.

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F_1 was purified as described by Klein et al. [4]. It was stored in 2.1 M ammonium sulfate, 50 mM Tris-HCl, 250 mM sucrose, 2 mM EDTA and 4 mM ATP (pH 8.0), at 4°C. The F_1 suspension in ammonium sulfate was centrifuged and the pellet was rinsed with 250 mM sucrose, 50 mM Tris-acetate (pH 7.5) (STA buffer) in the presence of 50% ammonium sulfate, and then resuspended in STA buffer. The F_1 solution was desalted by passing through an ACA 202 column (IBF) equilibrated with STA buffer, and then through another ACA 202 column equilibrated in 50 mM Tris base, 50 mM Mes, 1 mM $MgCl_2$ (pH 7.5) (TMMg buffer).

2.2. Azido-[α-^{32}P]PP_i photolabelling of isolated mitochondrial F_1-ATPase

A sample of 10 μM desalted F_1 (35 mg in 10 ml) was supplemented with 25 μM azido-[α-^{32}]PP_i and incubated for 30 min in darkness at room temperature in a small Petri dish. The photolabelling step consisted in two sequential photoirradiations of 30 s each, using a Xenon XB100 lamp (1000 W). The enzyme solution was protected from deleterious short-wavelength radiations by a glass plate placed between the light source and the Petri dish [5]. Under these conditions, photoirradiation of F_1 in the absence of photoprobe had no deleterious effect on its ATPase activity. Photolabelled F_1 was precipitated with 65% ammonium sulfate. The precipitate was recovered by centrifugation and resuspended in a medium consisting of 50 mM sodium succinate, 1 M sodium chloride, 0.25 M sodium nitrate, 0.1 mM dithiothreitol, and 4 mM EDTA (pH 6.1). Upon this treatment, the β-subunit was dissociated from F_1 and separated from the $\alpha\gamma\delta\epsilon$ complex by chromatography on DE-52 cellulose (Whatman) using a linear LiCl gradient [6].

2.3. Chemical and enzymatic cleavage of α- and β-subunits

The isolated β-subunit and the $\alpha\gamma\delta\epsilon$ complex were succinylated and then subjected to CNBr cleavage and proteolytic digest. CNBr cleavage was performed in 80% formic acid for 6 h at room temperature in the dark with a tenfold excess of CNBr (w/w). The CNBr peptides were first separated by gel filtration on a G75 (SF) column (100×2 cm). Full resolution was achieved by reverse-phase HPLC using a Vydac TP C-4 column (4.6×250 mm). A single photolabelled CNBr peptide was recovered from the subunit, whereas two distinct major photolabelled CNBr peptides were obtained from the $\alpha\gamma\delta\epsilon$ complex. The labelled CNBr peptides were further cleaved by proteolytic enzymes. Tryptic cleavage was carried out in 100 mM NH_4HCO_3, for 6 h at 37°C, 1/50 (w/w). In the case of cleavage by *S. aureus* V_8 and Endo Asp-N proteinases, incubation lasted 16 h at 37°C, 1/20 (w/w), using as buffers 100 mM NH_4HCO_3 and 100 mM Tris-HCl (pH 7.5), respectively. The products were separated by filtration on a Bio-Gel P4 column (1.5×80 cm).

2.4. Sequence analysis of the photolabelled peptides

The purified peptides were coupled to the arylamine groups of modified Sequelon membranes by their C-terminal carboxyl group and Asp and Glu carboxyl groups as described in [7]. The amino acid sequence of the coupled peptides was analyzed in an Applied Biosystems sequenator (model 477A). [^{32}P]P_i elution was optimized by extensive washing of the Sequelon membrane with methanol and TFA after each TFA cleavage step to avoid excessive carry-over of [^{32}P]P_i from one cycle to the next. Radioactivity of the eluted fractions from each sequencing cycle was determined by liquid scintillation counting.

2.5. PAGE

As resolution between the α- and β-subunits of F_1 was needed, TDAB-PAGE was used [8]. For peptide analysis, the electrophoresis system described by Schägger and von Jagow [9] was used. The peptides were then transferred on a Problott membrane (Applied Biosystems) with the Milliblot-Graphite Electroblotter Systems (Millipore), using 10 mM CHAPS and 20% (v/v) methanol as buffered medium. The current setting was 4 mA/cm^2 for 30 min.

3. Results

3.1. Effect of the concentration of mitochondrial F_1 on the photolabelling of the α- and β-subunits

In a preliminary report, it was shown that both the α and β-subunits of beef heart mitochondrial F_1 were covalently labelled following photoirradiation of the enzyme in the presence of azido-[^{32}P]PP_i [3]. Further experiments revealed that the label distribution in the α- and β-subunits varied upon F_1 concentration. When F_1 was used at concentrations of 1 μM and 10 μM, whereas azido-[α-^{32}P]PP_i was present in the incubation medium at the concentration of 25 μM, the yield of photolabelling was in the range of 18–20% for both concentrations. However, as revealed by TDAB-PAGE followed by autoradiography, the probe was covalently bound to the β-subunit to an extent of 70% when the concentration of F_1 was 1 μM, whereas at the higher F_1 concentration (10 μM) only one third of the probe was recovered in the β-subunit, the remaining being localized in the α-subunit (data not shown). The dependence on F_1 concentration of the photoprobe distribution between F_1-subunits might reflect the localization of the azido-[^{32}P]PP_i binding sites at the interface between the α- and β-subunits.

In photolabelling experiments described hereafter, F_1 and azido-[α-^{32}P]PP_i were used at the concentrations of 10

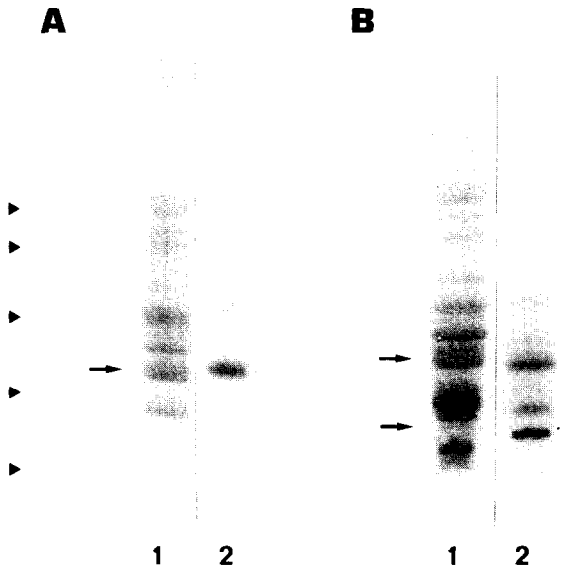

Fig. 1. SDS-PAGE of the CNBr digests. (A) SDS-PAGE of a CNBr digest of photolabelled β-subunit. Conditions for electrophoresis were the same as those described by Schägger and Von Jagow [9]. Lane 1, Coomassie brilliant blue staining; lane 2, autoradiography. (B) SDS-PAGE of a CNBr digest of photolabelled αγδε complex. Lane 1, Coomassie brilliant blue staining; lane 2: autoradiography. The predominantly labelled bands are designated by arrows. Molecular weight markers (arrowheads) have the following molecular masses: 16.9 kDa, 14.4 kDa, 8.2 kDa, 6.2 kDa and 2.5 kDa.

μM and 25 μM, respectively. Under these conditions, before exposure to light, about 1.5 out of the 2 high affinity azido-[α-^{32}P]PP_i binding sites of F_1 are occupied by the probe. The photoactivation results in the covalent binding of an average of 0.3 mol of azido-[^{32}P]PP_i per mol of F_1, with one third of the label present in the β-subunit and the remaining in the α-subunit.

3.2. Mapping of azido-[α-^{32}P]PP_i binding sites on the β-subunit of mitochondrial F_1

The CNBr digest of the photolabelled β-subunit was resolved by SDS-PAGE. All the covalently bound radioactivity was localized in a single band migrating with an apparent molecular mass of 7 kDa (Fig. 1A). This labelled peptide in the CNBr digest was recovered after fractionation of the digest by gel filtration on Sephadex G75 followed by reverse-phase HPLC (data not shown), essentially as described in [5]. It was identified by amino acid sequencing as a 7260 Da fragment of the β-subunit that extended from Gln-293 to Met-358, corresponding to the CNBr peptide CB9 (nomenclature of Runswick and Walker, [10]). Cleavage of β-CB9 by trypsin yielded two subfragments. The first one, referred to as β-R18 spanned Ile^{296}-Arg^{337} (4260 Da); the second one, β-R20, spanned Ala^{338}-Arg^{356} (2090 Da). The two tryptic peptides were resolved by gel filtration. Both were radiolabelled, with β-R18 carrying about two thirds of the total radioactivity of the β-subunit. Each peptide was attached to a Sequelon arylamine membrane, and sequenced (see Section 2). Bound radioactivity was found to be predominantly located on Tyr-311 of peptide β-R18, and on Tyr-345 of peptide β-R20 (Figs. 2A and B). Smaller amounts of radioactivity were found on Lys-301 and Gln-308 of peptide β-R18.

3.3. Mapping of the azido-[α-^{32}P]PP_i binding sites on the α-subunit of mitochondrial F_1

The photolabelled αγδε complex, obtained after dissociation from the β-subunit, was succinylated and cleaved in its entirety with CNBr. SDS-PAGE of the CNBr digest

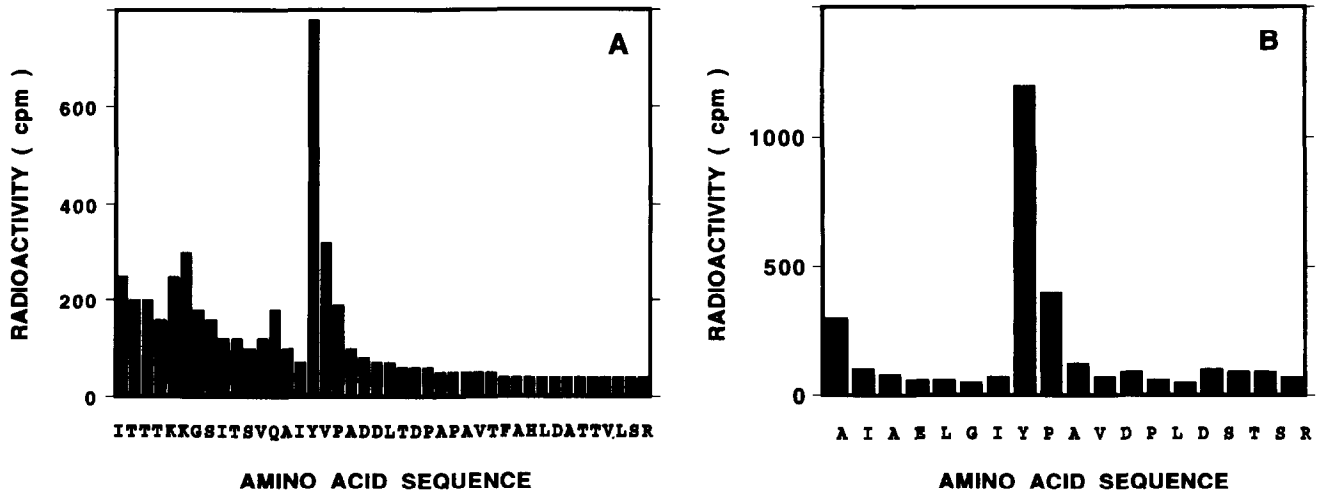

Fig. 2. Identification of the radiolabelled amino acid residues of the β-subunit. (A) Radioactivity elution from the Edman degradation of radiolabelled peptide β-R18. A fraction of the peptide was covalently coupled to a Sequelon™ aryl amine membrane (see Section 2). Several washes with TFA and methanol were carried out after each cleavage step to allow better extraction of radioactivity. Radioactivity was counted after evaporation of the volatile components. The amino acid sequence of the peptide is indicated along the *X*-axis. (B) Radioactivity elution of the Edman degradation of radiolabelled peptide β-R20. Same conditions as in (A).

followed by autoradiography revealed the presence of two major radiolabelled peptides with molecular masses of 7–9 kDa and 5–6 kDa (Fig. 1B). After electrotransfer, two CNBr peptides were identified in equal amounts by N-terminal sequence analysis of the band in the 7–9 kDa region, namely the α-CB3 fragment spanning Gln^{147}-Met^{226} and a tandem peptide αCB5-CB6 spanning Gly^{254}-Met^{312}. The labelled 5–6 kDa peptide detected by autoradiography had no corresponding stained band in the gel (Fig. 1B). Its amino acid sequence corresponded to that of a single peptide spanning Lys^{384}-Met^{436} corresponding to the tandem peptide αCB8-CB9.

For a more precise assignment of the binding sites of azido-[α-^{32}P]PP_i on the α-subunit, the components of the CNBr digest were resolved by gel filtration under the same conditions as those used for the peptides of the β-subunit. Bound radioactivity was recovered in two major peaks referred to as A2 and A3 (Fig. 3). Reverse-phase HPLC of the protein material contained in peak A2 allowed the recovery of a major radiolabelled purified peptide (Fig. 4A). This peptide was sequenced and identified as αCB3. The purified αCB3 peptide was further cleaved by endo Asp-N proteinase and the cleavage products were analyzed by SDS-PAGE, followed by autoradiography. The radiolabelled peptide was electrotransferred on a Problot membrane and sequenced. It was identified as the fragment spanning αAsp^{194}-Thr^{221}.

The radioactive material contained in the peak A3 (Fig. 3) was purified by reverse-phase HPLC (Fig. 4B). In agreement with previous identification after electrotransfer and sequence analysis (see above), N-terminal sequence analysis revealed that the radioactivity was associated with the tandem peptide αCB8-CB9. Attempts to localize the labelled amino acid residues in this peptide were unsuccessful because of difficulties encountered during further subdigestion of the isolated peptide.

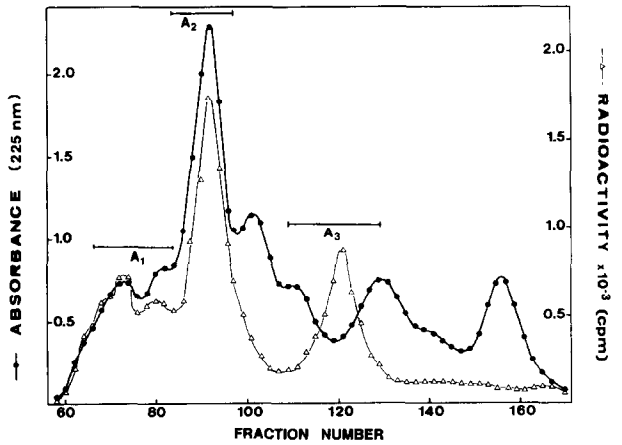

Fig. 3. Fractionation of the CNBr digest of the photolabelled $\alpha\gamma\delta\epsilon$ complex. The resulting peptides were separated by chromatography on a Sephadex G75 superfine column (120×2 cm) equilibrated in 50 mM ammonium bicarbonate. During elution, 3 ml fractions were collected and analyzed for absorbance at 225 nm and radioactivity (10 μl aliquots were counted). The bars (noted A1 to A3) correspond to pooled fractions. The recovery of the radioactive material was about 90%.

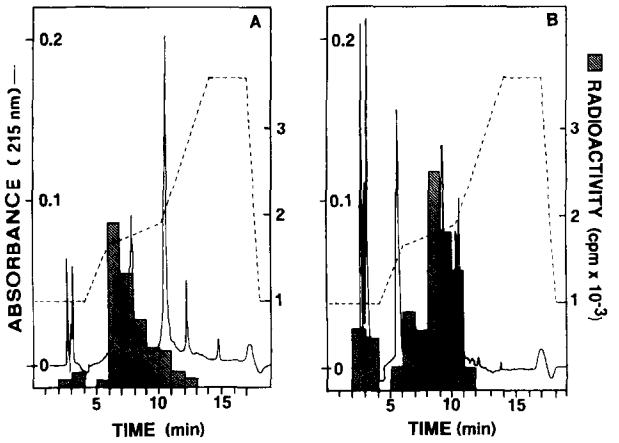

Fig. 4. Purification of the radiolabelled peptides obtained by CNBr cleavage of the $\alpha\gamma\delta\epsilon$ complex. The peptides were recovered, from Sephadex chromatography of a crude CNBr digest, as fractions A2 and A3 (see Fig. 3). They were purified by HPLC on a C4 column (10 μm, 4.6×200 mm, Vydac) equilibrated in 75% buffer A (0.1% TFA) and 25% buffer B (0.1% TFA, 80% CH_3CN). (A) The material corresponding to the pooled A2 fractions was injected. Fractions of 10 μl were collected at 1 min intervals, and analyzed for their radioactivity content. Most of the radioactivity was eluted in fraction 7. When the radioactive material present in fractions 8 and 9 was reinjected under the same conditions onto the HPLC column, the same radioactive profile was found with the main part of the radioactivity eluted in fraction 7. Thus, for the identification of the radiolabelled peptide, N-terminal sequence analysis was performed on the peptide present in fraction 7. (B) The material corresponding to the pooled A3 fractions was injected. The radiolabelled peptide was present in fractions 9 and 10. The dotted line is a schematic representation of the acetonitrile gradient used (% of buffer B).

4. Discussion

PP_i behaves as a diphosphate analog and binds to three sites on mitochondrial F_1 [1]. The three PP_i binding sites do not result from simple occupancy of empty nucleotide binding sites [2] as shown by the fact that the same number of PP_i binding sites are titrated in different forms of F_1 (F_1 [2,1], F_1 [0,0], F_1 [3,0], F_1 [2,2], where the first and second numbers designate the number of nucleotides bound to the non-catalytic sites and catalytic sites, respectively [11]). Incubation of F_1 with azido-[α-^{32}P]PP_i in the dark under conditions of saturation results in the reversible binding of the photoprobe to three sites, two of which exhibit a high affinity (K_d = 2 μM), the third one having a lower affinity (K_d = 300 μM) [3]. Azido-[α-^{32}P]PP_i mimics PP_i and behaves like ADP in a number of its binding properties. In particular, ADP and PP_i compete efficiently with azido-[α-^{32}P]PP_i for binding to mitochondrial F_1, but P_i and azido-P_i do not. Furthermore, an efficient protection of F_1 against photolabelling by azido-[α-^{32}P]PP_i is provided by PP_i and ADP [3]. Upon photoirradiation, azido-[^{32}P]PP_i binds to

both the α- and β-subunits of F_1, resulting in loss of the ATPase activity [3].

It was suggested that the binding sites for PP_i, and also azido-[^{32}P]PP_i, are located at the interface between the α- and β-subunits [3]. This idea fits with the finding that, at a fixed concentration of azido-[^{32}P]PP_i (25 μM), the distribution of the probe between α and β-subunits depends on the concentration of F_1. One may in fact imagine that the α and β subunits of F_1 contain PP_i binding sites differing by their affinity for PP_i, and that those sites are located at the α/β interface. Under the conditions used, the distribution of the covalently bound probe between the α- and β-subunits would reflect the respective affinities of the α-site and the β-site for azido-[^{32}P]PP_i and would therefore vary with the azido-[^{32}P]PP_i/F_1 ratio.

The present work was aimed at mapping the PP_i-binding sites of mitochondrial F_1 with azido-[^{32}P]PP_i under experimental conditions where the photoprobe binds to the two high affinity sites. Photolabelled α- and β-subunits were digested by CNBr and proteolytic enzymes, and the radioactive fragments were identified by amino acid sequence analysis. Three peptides were identified, namely βCB9 (Gln^{294}-Met^{358}) with maximal labelling carried by Tyr-311 and Tyr-345, αCB3 (Gln^{148}-Met^{227}) with most of the radioactivity concentrated in the sequence Asp^{194}-Thr^{221}, and a tandem peptide αCB8-CB9 (Lys^{386}-Met^{437}).

The polydisperse character of the photolabelling has to be explained. As β-Tyr-311 and β-Tyr-345 residues are located in peptide segments to which ADP and ATP bind [7,12–14], and are thought to be constitutive parts of the F_1 catalytic sites (for reviews, see [15–17]), one may infer from our results that the PP_i binding sites of F_1 contain amino acid residues belonging to the F_1 catalytic site. In agreement with previous studies carried out on the binding of PP_i to F_1 [2,18,19], the labelling of F_1 by azido-[^{32}P]PP_i does not seem to be restricted to the catalytic site of the enzyme as it is the case for the labelling by azido-[^{32}P]P_i, a phosphate analog [5]. As a matter of fact, the α-Asp^{194}-Thr^{221} and the α-Lys^{386}-Met^{437} regions, which are the major target sites of azido-[^{32}P]PP_i on the subunit, were never reported to be part of the F_1 catalytic site.

After our manuscript was submitted, the structure at 2.8 Å resolution of F_1 from bovine heart mitochondria was reported by Walker's group [20], providing evidence for the localization of the nucleotide binding sites at the interface between the α- and β-subunits, with the catalytic sites being predominantly in the β-subunits and the non-catalytic sites in the α-subunits. β-Tyr-345, which is photolabelled by azido-[^{32}P]PP_i and which was previously found to form a cross-link with 2-azido-ADP [7,12], is one of the residues which define the adenine- and ribose-binding pocket of the catalytic site of F_1 [20]. On the other hand, α-Tyr-203, α-Gln-208 and α-Gln-432 which belong to the α-Asp^{194}-Thr^{221} and α-Lys^{386}-Met^{437} peptides which are photolabelled by azido-[^{32}P]PP_i, contribute to the formation of the non-catalytic site of F_1 [20]. β-Tyr-311, an other amino acid residue which is photolabelled by azido-[^{32}P]PP_i, has been previously reported to be photolabelled by 8-azido-ADP [7,13] and azido-P_i [5]. However, the 3D-structure of F_1 indicates that β-Tyr-311 is at the opposite end of the binding site to the adenine pocket in the catalytic site of F_1, excluding apparently a productive binding of nucleotide to β-Tyr-311 [20]. A possible explanation for the dilemma concerning the photolabelling of β-Tyr-311 stems from earlier experiments dealing with the effect of fluoroaluminate on the photolabelling of F_1 by 8-azido-ADP. It was found that, in the presence of fluoroaluminate, the covalent binding of photoactivated 8-azido-ADP shifts from β-Tyr-311 to β-Tyr-345 [7]. In fact, the nucleotide-fluoroaluminate complex strongly binds to the catalytic site of F_1 which undergoes a rearrangement resulting in a conformation close to the transition state of the enzyme during the normal course of catalysis [21]. This suggests that the peptide segment encompassing β-Tyr-311 is endowed with some flexibility, which could explain its accessibility by the photoprobes despite its unfavourable position in the crystal structure.

These considerations help clarify the interpretation of previous results from this laboratory which showed that PP_i competed with ADP for binding to F_1 [3] and that the total number of moles of bound nucleotides and PP_i per mole of F_1 was less or equal to six [2]. Experimental evidence led us to postulate that PP_i could interact with both the catalytic and the non-catalytic nucleotide binding sites of F_1 [2]. The photolabelling experiments reported here are in full agreement with this statement, and they provide, at the light of the structural data, a realistic interpretation of the implication of PP_i as ligand of the catalytic and non-catalytic adenine nucleotide binding sites of F_1.

Acknowledgements

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